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(54) Title: METHOD FOR PRODUCING LARGE LATENT TRANSFORMING GROWTH FACTOR-β COMPLEXES AND LARGE LATENCY ASSOCIATED PEPTIDE

(57) Abstract

This invention generally provides for a method of producing recombinant large latent TGF-β by introducing nucleic acid sequences coding for LTBP and pro-TGF-β into eukaryotic cells. This invention further provides for a method for producing recombinant LL-TGF-β by introducing a DNA sequence for LTBP to an eukaryotic cell which expresses pro-TGF-β. The invention also provides for the construction of a pME-TGF-β2 plasmid and its co-transfection with pRSVneo plasmid into BP-1-1 CHO cells which contains an amplified LTBP cDNA sequence in the genome. The invention also provides for a method for treating the LL-TGF-β1 complex produced to bind to Ca^{2+} ions during the purification process activity. An antibody to the large latent TGF-β1 complex is also disolosed herein. Additionally isolated L-LAP and a method to produce the same is provided herein.

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METHOD FOR PRODUCING LARGE LATENT TRANSFORMING GROWTH FACTOR-B COMPLEXES AND LARGE LATENCY ASSOCIATED PEPTIDE

RELATED APPLICATION

This application is a continuation-in-part application of Serial No. 07/966,301, filed on October 26, 1992.

FIELD OF THE INVENTION

The invention relates to transforming growth factor-ß (TGF-ß). Specifically, the invention relates to the expression of large latent TGF-ß complexes in eukaryotic cells, such as Chinese Hamster Ovary (CHO) cells. The expression of the large latent TGF-ß (LL-TGF-ß) complexes in eukaryotic cells and the subsequent purification of the complexes are described herein. The invention further relates to a method for isolating large latency associated peptide (L-LAP) from rLL-TGF-ß as described herein.

BACKGROUND OF THE INVENTION

Transforming growth factor-ßs are a family of proteins with potent cellular modulating activities on many types of cells. See, e.g., Roberts and Sporn, Peptide Growth Factors and Their Receptors I, 419-472, 1990. Three human isoforms of TGF-ßs have been identified and characterized, TGF-ß1, -ß2, -ß3. TGF-ß1 was first identified as a growth factor which stimulated some rodent fibroblasts to grow in semi-solid agar. It is becoming clear, however, that TGF-ß1 is also a potent growth inhibitor for many different cell types, a modulator of

cellular differentiation, and an inducer of extracellular matrix production and deposition.

TGF-ß1 is produced by a wide variety of normal and malignant cells as a latent complex of high molecular weight. The structure of latent TGF-ß1 has been determined after purification from human platelets, Miyazono et al., J. Biol. Chem. 263, 6407-6415, 1988; Wakefield et al., J. Biol. Chem. 263, 7646-7654, 1988, and rat platelets, Okada et al., J. Biochem. 106, 304-310, 1989. Its biosynthesis has been characterized by using a human erythroleukemia cell line, Miyazono et al., EMBO J. 10, 1091-1101, 1991.

The latent form of TGF-ß1 consists of three distinct components: 1) mature TGF-ß1; 2) an N-terminal remnant of the TGF-ß1 precursor; and 3) the latent TGF-ß1 binding protein (hereinafter referred to as "LTBP"). The N-terminal remnant of the TGF-ß1 precursor is important for TGF-ß1 latency, so it has been denoted TGF-ß1 latency associated peptide (ß1-LAP). Gentry et al., Biochemistry, 29, 6851-6857, 1990. The large latency associated peptide (rL-ß1-LAP), as designated herein, consists of two components; the TGF-ß1 latency associated peptide (ß1-LAP) and LTBP.

Mature TGF-ß1 is a disulfide-bonded dimer which has been proteolytically cleaved from ß1-LAP, which forms a disulfide-bonded dimer linked to a single molecule of LTBP. The latent TGF-ß1 complex which includes LTBP is referred to as the "large latent complex (LL-TGF-ß1)" whereas the complex without LTBP is the "small latent complex". Wakefield et al., Growth Factors, 1, 203-218, 1989; Miyazono et al., EMBO J., 10, 1091-1101, 1991.

LTBP was first purified from human platelets as a free form and as a component of the LL-TGF-ß1 complex. A cDNA clone coding for LTBP was recently isolated from human foreskin fibroblasts. See U.S. Serial No. 07/487,343, U.S. Patent No. 5,177,197, the contents of which are, incorporated herein by reference in its entirety. The open reading frame of the cDNA sequence predicted that LTBP is

a 1394 amino acid protein containing two different types of repeat sequences; sixteen epidermal growth factor (EGF)-like repeats and three copies of a repeat sequence containing eight cysteins in one motif. In some of the EGF-like repeat sequences, ß-hydroxylated asparagine residues were identified, Kanzaki et al., Cell 61, 1051-1061, 1990, indicating that LTBP is a Ca^{2+} binding protein, Dahlback et al., J. Biol. Chem. 266, 18481-18489, 1990. LTBP is not directly associated with TGF-ß1 latency, but it plays a role in the assembly and secretion of latent TGF-ßi molecules by producer cells.

Substantially pure, receptor like TGF-ß1 binding glycoproteins have been found. These molecules are characterized by molecular masses of 160 kD, 70-80 kD, and 30-40 kD as determined by SDS-PAGE and the ability to bind the TGF-ß1 molecule. See U.S. Serial No. 07/717,316, U.S. Patent No. 5,229,495, the contents of which are incorporated herein by reference in its entirety.

SUMMARY OF THE INVENTION

This invention generally provides for a method of producing recombinant large latent TGF-ß by introducing DNA sequences coding for LTBP and pro-TGF-ß into eukaryotic cells. These sequences are introduced to the eukaryotic cells and co-expressed. As used herein, the terms "co-expression" and "co-expressed" mean expression of the two components of the complex, i.e., pro-TGFß and LTBP, by any of the means known to a skilled artisan. These means may include, e.g., sequential transformation using two expression vectors, or transforming a single vector capable of expressing both components.

This invention further provides for a method for producing recombinant large, latent TGF-ß by introducing DNA sequence for LTBP into a eukaryotic cell which expresses pro-TGF-ß. Specifically, the invention provides for the construction of a LTBP expression plasmid, pDSVE2-BP, its subsequent transfection into CHO cells, the CHO

cell, T23-7-11, is particularly preferred. The clone with the highest LL-TGF-ß complex expression was chosen for purification (LT3-1 clone).

The invention also provides for the construction of a pME-TGF-ß2 plasmid and its co-transfection with pRSVneo plasmid into BP-1-1 CHO cells which contains an amplified LTBP cDNA sequence in the genome. Expression of LL-TGF-ß2 activity into culture medium is assayed.

Also provided herein is a method for isolating L-LAP by degrading LL-TGF-ß and separating L-LAP therefrom.

An antibody which specifically binds to the LL-TGF-ß complex but not to LTBP is also described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Construction of the expression plasmid pDSVE2-BP.

Figure 2A Detection of LL-TGF-ß1 secreted from cell clones transfected by plasmid pDSVE2-BP into culture medium using Ab-39 polyclonal antibody.

Figure 2B Experiment described in Figure 2A, using LT-1 polyclonal antibody

Figure 3 Purification scheme of the LL-TGF-ß1 complex from LT3-1 conditioned medium.

Figure 4A Analysis of purified LL-TGF-ß1 complex on SDS-PAGE.

Figure 4B Analysis of purified LL-TGF-ß1 complex after SDS-PAGE by immunoblotting.

Figure 5 Separation and sequencing of proteolytic fragments of purified LTBP.

Figure 6 Inhibition of CCL-64 cell DNA synthesis.

Figure 7 Analysis of recombinant LTBP eluted from C4 reversed phase column by SDS-PAGE

Figure 8 Detection of LL-TGF-β2 secreted from BP-1-1 cell clones transfected with plasmid DNA containing a prepro-TGF-β2 cDNA.

Figure 9 $^{45}Ca^{2+}$ binding to LTBP.

Figure 10A Effect of Ca^{2+} on the susceptibility to trypsin of the free form LTBP.

Figure 10B Effect of Ca^{2+} on the susceptibility to trypsin of the recombinant LL-TGF-β1.

Figure 11A Effects of Ca^{2+} on LTBP anion exchange chromatography on LTBP in the presence of 5mM EDTA.

Figure 11B Experiment described in Figure 11A, using 5mM $CaCl_2$.

Figure 12 Purification of recombinant L-LAP by gel filtration on Superose 12.

Figure 13 Analysis of purified recombinant L-LAP by SDS-PAGE.

DETAILED DESCRIPTION OF THE INVENTION

This invention will be better understood by reference to the following examples, which are included here for purposes of exemplification and are not to be construed as limitations.

Example 1

An expression plasmid for LTBP, named pDSVE2-BP, was constructed utilizing the standard recombinant methods such as described in Maniatis et al., Molecular Cloning: A

Laboratory Manual, 1982. Specifically, the plasmid pDSVE2-BP was constructed as follows: pUC19-A and pUC19-B are plasmids carrying the 5'-half EcoRI fragment of an LTBP cDNA with the 5' untranslated region (Kanzaki et al., Cell 61, 1051-1061, 1990) and the 3'-half EcoRI fragment of the LTBP cDNA with the 3' untranslated region, respectively. First, a DraI-EcoRI fragment carrying the 5' half of LTBP cDNA was isolated from pUC19-A and subcloned into the PstI-EcoRI site of SRα-296 plasmid (Takabe et al., Mol. Cell. Biol. 8, 466-469, 1988). The 3'-half EcoRI fragment was then isolated from pUC19-B and introduced into the EcoRI site of this SRα-296 plasmid to construct SRα-BP, a plasmid carrying the full-length cDNA of LTBP. Finally the LTBP cDNA was isolated from SRα-BP by XhoI cleavage and inserted into the SalI site of a mammalian expression plasmid pDSVE2 containing a mouse dihydrofolate reductase (dhfr) minigene for gene amplification. The final plasmid was named pDSVE2-BP, see Figure 1.

Example 2

T23-7-11 is a CHO cell line which produces a large amount of recombinant human pro-TGF-β1 complex. The construction and the properties of this cell line is described in detail in a laid-open Japanese patent application (KOKAI 3-180192, 1981) which is hereby incorporated by reference. The essential elements describing the construction of T23-7-11 cell line, however, are briefly described below.

Human prepro-TGF-β1 cDNA clone was isolated from a λgt10 cDNA library of human placenta cells. Total RNA was prepared from human placental tissue and poly(A) RNA was isolated by oligo(dT)- cellulose column chromatography using methods well known in the art. Double-stranded cDNA was synthesized by the method of Gluber and Hoffmann (Gluber et al., Gene 25, 263, 1983) and cloned into the EcoRI site of a λgt10 vector. An oligo DNA probe was synthesized according to the published nucleotide sequence

of human TGF-ß1 cDNA (Derynck et al., Nature 316, 701-705, 1985). Clones carrying the cDNA sequence for human prepro-TGF-ß1 protein were screened from the human placenta library using this probe. Using plaque hybridization and DNA sequencing, one cDNA clone, T1, carrying a full-length cDNA sequence for human prepro-TGF-ß1 was identified from 3×10^5 independent plaques. This cDNA clone was then subcloned into the EcoRI site of pUC19.

A mammalian expression plasmid for human prepro-TGF-ß1 cDNA, pECß, was constructed as follows. First, the pUC-19 plasmid carrying the human prepro-TGF-ß1 cDNA was digested with EcoRI. An isolated EcoRI fragment containing the cDNA was made blunt-ended and then inserted into the SmaI site of vector pSVL to obtain pSVL-TGF-ß1. Second, the mammalian expression plasmid CDM8 (Seed, B., Nature 329, 840-842, 1987) was digested with SacII and BamHI. The isolated SacII-BamHI fragment containing an amber suppressor supF tRNA gene, the cytomegalovirus (CMV) immediate early promoter DNA sequence and the Simian Virus 40 (SV40) polyadenylation DNA sequence was made blunt-ended and then introduced into the PvuII site of vector pSV2dhfr (Subramani, S. et al, Mol. Cell. Biol. 1, 854-864, 1981) to obtain pCMV-dhfr. Finally, the XbaI-EcoRI fragment of pSVL-TGF-ß1 (containing the human prepro-TGF-ß1 cDNA, the SV40 polyadenylation sequence, an ampicillin-resistant gene and the ori derived from pBR322 DNA) and the XbaI-EcoRI fragment of pCMV-dhfr (containing the CMV promoter sequence and a gene cassette consisting of the SV40 early promoter, mouse dihydrofolate reductase (dhfr) coding sequence and the SV40 polyadenylation sequence) were ligated together. The resulting expression plasmid, pECß, carries the CMV promoter, the SV40 polyadenylation signal for human prepro-TGF-ß1 expression, the SV40 early promoter and the SV40 polyadenylation signal for mouse dhfr expression.

For the expression of human pro-TGF-ß1 protein, a dhfr$^-$cell line, i.e. CHO-DUKX B11 (Chasin, L.A. and Urlaub, G., PNAS USA 77, 4216-4220, (1980) was used. An

equivalent dhfr$^-$ CHO cell line is publicly available from the ATCC as ATCC CRL 9096 and could be used in place of CHO-DUKX B11. Cells were cultured in MEMα(-) supplemented with 10% fetal calf serum (FCS). The cells were transfected with a calcium-phosphate co-precipitate of pECß plasmid DNA and selected in the same medium containing 15% dialyzed fetal calf serum (FCS). The transformants were further cultured in the presence of 500 nM of methotrexate for gene amplification. The presence of human pro-TGF-ß1 in the conditioned media of transformants was detected by the growth inhibition assay of CCL-64 cells as described in detail in Example 6 and immunoblotting using a rabbit polyclonal antibody raised against pro-region of TGF-ß1 purified from human platelets (Kanzaki, et al., supra.). The cell clone with the highest TGF-ß1 activity in the conditioned medium was selected and named T23-7-11.

The T23-7-11 cell line has been deposited under the Budapest Treaty in Fermentation Research Institute (Japan) at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305 Japan, as FERM BP-4024 on October 7, 1992.

Example 3

The T23-7-11 cells described in Example 2, supra, were cultured in Ham's F-12 and Dulbecco's Modified Eagle's medium (1:1) supplemented with 15% FCS and 500nM methotrexate. The cells were transfected with the plasmid pDSVE2-BP constructed as described above and plasmid pSV2neo, using standard electroporation methods. Typically, a high concentration of plasmid DNA containing a cloned gene is added to a suspension of cells and the mixture is shocked with an electrical field of 200-600 V/cm. The brief electric pulse is discharged across the electrodes, transiently opening holes in cell membranes. Successfully transfected clones were then selected in the same medium containing 1mg/ml G418 and 500nM MTX. Seven clones resistant to G418 and 500nM MTX were picked and assayed for the secretion of LL-TGF-ß1 complex.

The clones were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques by the following procedure. After cell clones resistant to G418 were cultured to confluence in culture medium containing 10% FCS, they were rinsed twice with PBS and incubated for 4 days in serum-free medium containing 5 mg/l bovine insulin. After 4 days, conditioned medium was collected and concentrated 5 fold by ultrafiltration. These samples were subjected to SDS-PAGE on 4-12% gradient gel under non-reducing conditions, and then blotted onto PVDF membranes in a buffer containing 20% methanol, 192mM glycine and 25mM Tris-HCl buffer, pH 8.4 at 100V. The PVDF membranes were blocked by incubation with Tris-HCl buffer, at pH 7.4, containing 1% degraded gelatin, 0.1% Tween 20 and 150mM NaCl, and then incubated with alkaline phosphatase-conjugated antibodies (Ab39, LT-1) in the same buffer for 16 hours at 4°C. The PVDF membranes were then washed and visualized using NBT/BCIP substrate precipitation. Figure 2 shows the results of the incubation with the Ab39 and LT-1 polyclonal antibody conjugates. These results show that all seven cell clones secreted LL-TGF-ß1 complex and pro-TGF-ß1 into serum-free culture medium. The LT3-1 clone secreted the highest levels of LL-TGF-ß1 into the culture medium and was selected as a candidate cell clone for the purification of LL-TGF-ß1 complex from conditioned medium.

Cell line LT3-1 has been deposited under the Budapest Treaty in Fermentation Research Institute (Japan) as FERM BP-4015 on September 29, 1992.

Example 4

To obtain a supply of recombinant LL-TGF-ß1, the LT3-1 cells were grown to confluence in roller bottles containing 200ml selective medium. The cultures were rinsed with phosphate-buffered saline (PBS) and incubated for 7 days in serum-free F-12/DMEM supplemented with insulin, transferrin, monoethanolamine, sodium selenite,

aprotinin and polyethylene glycol 6000 as growth-promoting supplements (200ml/bottle). After 7 days, 80 liters of conditioned medium were collected.

The purification scheme is shown, schematically, in Figure 3.

To elaborate, the eighty liters of conditioned medium collected were concentrated, and desalted using Millipore ultrafiltration membranes with a molecular cutoff range of 100kD. The concentrated conditioned medium was then fractionated on a Q-Sepharose Fast Flow cation-exchange chromatography column equilibrated with 10mM sodium phosphate buffer at pH 7.2. Bound protein was eluted using a 200-660mM NaCl gradient with a flow rate of 8.0 ml/min. The presence of the LL-TGF-ß1 complex was monitored by SDS-PAGE and immunoblotting using the rabbit polyclonal antibody Ab39.

Fractions containing the LL-TGF-ß1 complex were further processed on an HP-10 hydroxyapatite column (50x100mm) equilibrated with 10mM sodium phosphate buffer at pH 7.2. The unbound fraction was collected and loaded on a Sulfated Cellulofine column (30x100mm) equilibrated with 10mM sodium phosphate butter at pH 7.2. The bound proteins were eluted using a 0-200 mM NaCl gradient with a flow rate of 4.0ml/min. The fractions containing the LL-TGF-ß1 complex were pooled and dialyzed with 40mM Tris-HCl buffer at pH 8.0. The dialyzed sample was applied to a DEAE-Toyopearl anion-exchange column (15x100mm) equilibrated with 40mM Tris-HCl buffer at pH 8.0, and then eluted using a 0-250mM NaCl gradient with a flow rate of 2.0ml/min.

The fractions containing high concentrations of the LL- TGF-ß1 complex were pooled, concentrated using an Amicon YM100 ultrafiltration membrane, and then applied to a Sephacryl S-300HR gel filtration column (20x1000mm) equilibrated with PBS. The column was eluted in the same buffer at a flow rate of 0.5ml/min. The purified protein was analyzed by SDS-PAGE followed by silver staining and

immunoblotting as shown in Figure 4, which shows the purified protein from S-300HR was analyzed by SDS-PAGE on 4-12% gradient gel and silver staining in the presence or absence of dithiothreitol (panel A), and the purified protein also analyzed by immunoblotting using Ab39 (A) or LT-1 (B) antibodies in the presence or absence of dithiothreitol (panel B).

Under non-reducing conditions, purified LL-TGF-ß1 complex revealed two protein bands with an apparent molecular mass of 220 kD and 270 kD. Both bands are recognized by Ab39 and LT-1.

Under reducing conditions, these proteins were separated into four bands with apparent molecular masses of 12.5kD, 40kD, 53kD and 150-190kD. Protein bands of 40kD and 53kD were recognized by LT-1 indicating that these may contain ß1-LAP. Protein bands of 12.5kD and 53kD were recognized by rabbit polyclonal antibody Ab57 against the partial polypeptide of mature TGF-ß1, suggesting that these may contain a mature TGF-ß1 sequence. The protein band of 150-190 kD was recognized by Ab39. These results indicated that these proteins were composed of LTBP, pro-TGF-ß1, ß1-LAP, and mature TGF-ß1.

Example 5

The identity of recombinant LL-TGF-ß1 complex was assessed further by direct amino acid sequencing of each component under reducing conditions.

The test samples were subjected to SDS-PAGE and then transferred to a PVDF membrane. After electroblotting, proteins on the membrane were detected with Ponceau.S dye. The stained spots were cut out and the proteins purified from PVDF membrane in the presence of dithiothreitol and electroblotting.

The N-terminal amino acid sequencing of the 12.5kD component revealed the sequence: Ala-Leu-Asp-Thr-Asn-Tyr-X-Phe-Ser-Ser, which was identical to the sequence of mature TGF-ß1. The N-terminal amino acid sequence

analysis of the 40kD and 53kD components revealed the sequence: Leu-Ser-Thr-X-Lys-Thr-Ile-Asp-Met-Glu, which was identical to that of the precursor sequence of TGF-β1.

The attempt to determine the N-terminal amino acid sequence of the 160-190kD component was unsuccessful, indicating that the N-terminal may be blocked. Therefore purified LTBP was digested with Endoproteinase Asp-N and separated on a narrow-bore, reversed-phase HPLC column eluted with linear gradients of acetonitrile/2-propanol. The effluents were monitored at 215 nm. Amino acid sequence analyses were performed on the materials under the numbered peaks by using a SHIMADZU PSQ-1 protein sequencer. The sequences obtained were comparable to those described by Kanzaki et al., Cell 61, 1051-1061 (1990). The results of amino acid sequencing of three different polypeptides revealed the sequences: Asp-Ile-Asn-Glu-Cys-Leu-Glu (No. 10), Asp-Gln-Gly-Tyr-Arg-Ala-Ser (No. 12), Asp-Pro-Val-Lys-Leu-Gln-Cys-Leu (No. 18). See Figure 5. These sequences are identical to those of the internal sequences of LTBP except the Leu10 residue of No. 18 peptide, Kanzaki et al., Cell 61, 1051-1060, 1990. Thus, the 150-190kD component was determined to be LTBP.

The purified LL-TGF-β1 complex was found to be composed of four different subunits with molecular masses of 12.5 kD, 40 kD, 53 kD, and 150-190 kD, which were identified as mature TGF-β1, β1-LAP, pro-TGF-β1 and LTBP, respectively.

Example 6

TGF-β1 activity was determined using a ^{3}H-thymidine incorporation assay of mink lung epithelial cells, CCL-64, (Cone et al., Anal. Biochem, 168, 71-74, 1988). CCL-64 cells and the test samples were transferred into a 96 well tissue culture plate in DMEM supplemented with 10% FCS and antibiotics. After a 48 hour incubation, cells were pulsed with 0.5μCi of ^{3}H-thymidine for four hours. The ^{3}H radioactivity incorporated into DNA was

determined with a liquid scintillation counter. Purified protein was quantitated using a Bio-Rad protein assay kit. Figure 6 shows the biological activity profiles. Recombinant mature TGF-ß1 was a potent growth inhibitor of CCL-64 cells.

When recombinant LL-TGF-ß1 complex was activated by acidification, it was also a potent growth inhibitor of CCL-64 cells. Its specific activity, however, was approximately a fifth that of recombinant mature TGF-ß1 with half-maximal inhibition observed with 6 ng purified protein/ml. These results were similar to those obtained with LL-TGF-ß1 complex isolated from human platelets. Miyazono et al., J.Biol. Chem. 263, 6407-6415, 1988. Recombinant LL-TGF-ß1 complex was much less effective in inhibiting CCL-64 cells with half maximal inhibition at approximately 200 ng/ml and its inhibition curve appeared to have a slightly altered slope when compared with recombinant TGF-ß1 and acid-activated LL-TGF-ß1 complex. It was not previously reported that the natural LL-TGF-ß1 complex inhibited the proliferation of CCL-64 cells.

In figure 6, DNA synthesis inhibition was assessed as described below. Symbols: (○), recombinant mature TGF-ß1: (●) recombinant LL-TGF-ß1 complex (no treatment): (□) recombinant LL-TGF-ß1 complex (acid activated).

The tests performed indicate that the recombinant LL-TGF-ß1 complex secreted by LT3-1 cells appears identical to natural LL-TGF-ß1 complex derived from human platelets, indicating that LT3-1 cells represent a useful source in studies of the structure and functions of LL-TGF-ß1 complex. Moreover, recombinant LL-TGF-ß1 complex, recombinant small latent TGF-ß1 complex and recombinant mature TGF-ß1 which were produced by LT3-1 cells are useful in therapeutic and physiological studies, as discussed infra.

Example 7

A CHO cell line producing recombinant LTBP was established. To the extent of Applicants' knowledge, this is the first report of the stable production of LTBP at a moderate level using recombinant DNA methodologies.

For the expression of LTBP, the dhfr$^-$ cell line CHO-DUKX-B11, as described in Example 2, supra., was transfected with the LTBP expression plasmid pDSVE2-BP of Example 1. Cells were maintained in MEMα(-) supplemented with 5% FCS and transfected with 20 mg of pDSVE2-BP plasmid in a calcium phosphate co-precipitate. Dhfr$^-$ expressing transformants were selected by replacing the medium with MEMα(-) supplemented with 5% dialyzed FCS.

The presence of LTBP in the conditioned medium of each transformant was determined by immunoblotting. Samples were run on a 5-20% gradient SDS-polyacrylamide gel and blotted onto a nitrocellulose sheet. Nonspecific protein binding was blocked by incubating the sheet for 1 hr at room temperature in 0.05% Tween 20, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 5% dried milk powder. The nitrocellulose sheet was then incubated in the same buffer containing 10 mg/ml of anti-LTBP polyclonal antibody Ab-39. The sheet was washed twice in the same buffer and incubated with anti-rabbit IgG antibody conjugated with alkaline phosphate in the same buffer. The blots were washed and visualized using NBT/BCIP substrate precipitation.

Cell clones producing LTBP in the conditioned medium were selected and further cultured in the medium containing an increasing concentration of methotrexate up to 50 nM to amplify the introduced LTBP cDNA. One clone, BP1-1, was chosen for further study.

BP1-1 cells were grown to confluence in roller bottles containing 200 ml selective medium. The cells were rinsed with phosphate-buffered saline (PBS) and further incubated for 7 days in serum-free F-12/DMEM supplemented with insulin, transferrin, monoethanolamine, sodium selenite, aprotinin and polyethylene glycol 6000 as

growth-promoting supplements (200 ml/bottle). After 7 days, twenty liters of conditioned medium were collected.

The collected conditioned medium was concentrated and desalted using Millipore ultrafiltration membranes with a molecular cut off range of 10 kD. Concentrated conditioned medium was dialyzed against 25 mM sodium phosphate buffer containing 0.5 M ammonium sulfate at pH 7.2 and then applied to a Phenyl-Toyopearl 650M column (50 × 100 mm, Tosoh) equilibrated with the same buffer. The bound proteins were eluted using a 0.5-0 M ammonium sulfate gradient at a flow rate of 5.0 ml/min. The presence of LTBP was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and/or enzyme-immunoassay using the rabbit polyclonal antibody Ab-39. Fractions containing LTBP were pooled, dialyzed against 10 mM Tris-HCl buffer at pH 8.0 and further processed on a Q-Sepharose FF anion exchange column (26 × 100 mm) equilibrated with the same buffer. The bound proteins were eluted using a 100-600 mM NaCl gradient at a flow rate of 3.0 ml/min. Fractions containing LTBP were pooled, concentrated and loaded on Superose 12 prep grade column (26 × 500 mm) equilibrated with PBS. The loaded samples were eluted with PBS at a flow rate of 1.0 ml/min. Fractions containing LTBP were pooled and applied to a reversed-phase C4 HPLC column (11 × 250 mm) equilibrated with 10% acetonitrile containing 0.1% TFA, and then eluted using a 10-50% acetonitrile gradient at a flow rate of 2.0 ml/min.

Purified protein was separated by SDS-PAGE and analyzed by silver staining, immunoblotting using the anti-LTBP antibody Ab-39 as described above, and amino acid sequencing. Under both reducing and non-reducing conditions, the purified protein revealed a single broad band with an apparent molecular weight of 110-130 kD by silver staining. As shown in Figure 7, SDS-PAGE revealed a diffuse protein band with an apparent weight of about 120 to about 140 kDa. This band was recognized by the rabbit

anti-LTBP Ab-39 antibody, indicating that it was LTBP. The identity of the purified protein was further confirmed by amino acid sequencing under reducing conditions. The protein was digested with an endopeptidase Asp-N, subjected to a reversed-phase HPLC and the separated polypeptides were sequenced. The obtained amino acid sequences were identical to those found in the reported amino acid sequence of LTBP purified from human platelets (Kanzaki et al., Cell 61, 1051-1061, 1990).

Cell line BP1-1 was deposited under the Budapest Treaty in Fermentation Research Institute (Japan) as FERM BP-4014 on September 29, 1992.

Example 8

The production of LL-TGF-ß2 was achieved by co-expressing the LTBP cDNA with a prepro-TGF-ß2 cDNA in CHO cells. A prepro-TGF-ß2 expression plasmid was introduced to the BP1-1 cell line described above.

The complete nucleotide sequence of human TGF-ß2 has been published (De Martin et al., EMBO J. 6, 3673-3677, 1987). A cDNA clone for human TGF-ß2 could be obtained essentially as described for the TGF-ß1 cDNA in Example 2. A mammalian expression plasmid pME-TGF-ß2 was constructed using the obtained prepro-TGF-ß2 cDNA. This plasmid contains the human TGF-ß2 cDNA under the control of a modified SV40 promoter and the SV40 early region for transcription termination and polyadenylation.

pME-TGF-ß2 plasmid was co-transfected with pRSVneo plasmid, which allows for neomycin resistance selection, into the BP1-1 cell line by electroporation. G418 resistant transformants were selected and assayed for the production of TGF-ß2 activity by the growth inhibition assay of CCL-64 cells (see Example 6). Several cell clones were isolated which secreted TGF-ß2 activity into culture medium. The methodology by which these clones were identified is described in Example 9.

Example 9

To immunologically detect LL-TGF-ß2 in the conditioned medium of several transfectants for TGF-ß activity, the medium was concentrated with Centriprep 10 concentrator and then analyzed by SDS-gel electrophoresis, (4-20% polyacrylamide gradient gel). Subsequent immunoblotting using anti-human LTBP polyclonal antibody, Ab39, identified four molecular species varying in apparent molecular size: 100-110 kDA, 120-130 kDa, 150-160 kDa, 220-220 kDa, as shown in Figure 7.

The 200-220 kDa represents LTBP associated with pro-TGF-ß2, whereas the 100-110 kDA, 120-130 kDA and 150-160 kDA species may represent free LTBP protein because these species are found in conditioned medium of BP-1-1 cells. This result shows that BP-1-1 transfected with a plasmid containing cDNA coding for human prepro-TGF-ß2 protein secrete pro-TGF-ß2 associated with a LTBP, yielding a LL-TGF-ß2 complex. This also demonstrates that association of LTBP and pro-TGF-ß2 co-expressed in CHO cells occurs during synthesis of each protein.

A cell line producing LL-TGF-ß2 was chosen and named LT2-14. Cell line LT2-14 was deposited under the Budapest Treaty in Fermentation Research Institute (Japan) as FERM BP-4016 on September 29, 1992.

Example 10

It has also been found that LL-TGF-ß1 and LTBP are capable of binding Ca^{2+}, leading to methodologies for purifying and stabilizing LL-TGF-ß via combination with the cation. The methods disclosed below are further described in Colosetti et al., FEBS Letters 320: 140-144, (1993), which is incorporated by reference herein in its entirety.

Recombinant LL-TGF-ß1 (10µg, pretreated with different concentrations of EDTA or $CaCl_2$), human platelet LTBP (10µg) (isolated from a side fraction from the purification of LL-TGF-ß1 from human platelets, Kanzaki et al., Cell 61:1051-1061, 1990), recombinant small latent

TGF-ß1 complex (SLC; 10µg), and human blood (B; 2µl), were subjected to SDS-PAGE (4-10% polyacrylamide gradient), and then transferred to a nitrocellulose membrane. After overnight incubation with $^{45}CaCl_2$ (1µCi/ml), the membrane was rinsed, dried and subjected to autoradiography.

The experimental data presented in Figure 9, show that both free LTBP and LL-TGF-ß1 exhibit clear binding of $^{45}Ca^{2+}$. Recombinant small latent TGF-ß1 and a sample of human blood used as controls did not bind $^{45}Ca^{2+}$ although proteins were seen by Ponceau S. staining.

Example 11

In order to investigate possible effects of Ca^{2+} binding on protein structure, free LTBP isolated from the conditioned medium of human prostate cell line PC-3 labelled with [^{35}S] cysteine, were immunoprecipitated by Ab39 antibodies and subjected to proteolytic digestion using trypsin over different time periods at constant temperature of 37°C, in the presence of 2 mM $CaCl_2$ or 2mM EDTA.

The incubations were quenched by the addition of 2 µg of soybean trypsin inhibitor. Samples were analyzed by SDS-PAGE and fluorography. A protective effect of Ca^{2+} on LTBP was observed, i.e., a 20 fold longer incubation time in the presence of Ca^{2+} was needed to obtain the same degree of degradation as that obtained in the presence of EDTA. The size of the protease resistant fragments were similar (See Figure 10A).

Similar experiments were conducted with LL-TGF-ß1. Tryptic digestion was performed in the presence of 5 mM $CaCl_2$ or 5mM EDTA. One µg of recombinant LL-TGF-ß1 was incubated with trypsin in 50 mM Tris-HCl, pH 7.4, with 5 mM EDTA or $CaCl_2$. Samples were then analyzed with SDS-PAGE and silver staining. Virtually no digestion of LL-TGF-ß1 was observed in the presence of $CaCl_2$, whereas in the presence of EDTA, eight bands could be observed after 20 minutes of incubation with trypsin (See Figure 10B).

Example 12

The effect of Ca^{2+} on the LTBP molecule was also investigated by anion exchange chromatography in the absence or presence of EDTA or $CaCl_2$. Free LTBP obtained from PC-3 conditioned medium in the presence of 5 mM EDTA or 5mM $CaCl_2$ was subjected to Q-Sepharose chromatography.

Aliquots of PC-3 cell conditioned medium (15 ml) were combined with 5mM EDTA (A) or 5 mM $CaCl_2$ (B) and subjected to chromatography on a Q-Sepharose column equilibrated with 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, with 5mM $CaCl_2$ or EDTA, respectively at a flow rate of 150 ml/hr. A 150-600 mM NaCl gradient (45min) was used for elution. The elution position of LTBP was followed by monitoring absorbance at 280 nm and the analysis of aliquots of fractions with 5ml were analyzed by SDS-PAGE and immunoblotting using the Ab39 serum (See Figure 11).

When samples were analyzed without $CaCl_2$ or EDTA addition, LTBP mainly eluted at about 460 mM NaCl. In the presence of 5 mM EDTA, LTBP eluted later i.e., at about 500 mM NaCl. An earlier elution at about 420 mM NaCl was observed if Ca^{2+} was present. The lower affinity of LTBP for the anionic ion exchanger in the presence of Ca^{2+} could be due to a changed charge on LTBP after Ca^{2+} binding, or to a Ca^{2+} induced change in conformation of the molecule.

It was also observed that this effect of Ca^{2+} binding is reversible: if LTBP was chromatographed in the presence of EDTA or $CaCl_2$ and then rerun after addition of $CaCl_2$ or EDTA, a shift in elution position was observed.

Example 13

Large quantities of rL-ß1-LAP can be isolated from rLL-TGF-ß, which has been prepared according the procedure set forth in Example 3 above. The LL-TGF-ß complex can be treated with a conventional denaturing agent, e.g. urea, to dissociate L-LAP from the LL-TGF-ß complex.

L-ß1-LAP was prepared by first dialyzing one mg of rLL-TGFß with 20mM sodium phosphate buffer (pH of 7.2) containing 8M urea. The dialyzed protein was concentrated and then applied to a Superose 12 prep grade gel filtration column. The column was equilibrated with 20mM (pH 7.2) sodium buffer containing 8M urea and 500mM NaCl. The sample was then eluted with the same buffer. Figure 12 shows the purification of rL-ß1-LAP by gel filtration on Superose 12. The separation proceeds based on the relative size of the two populations. The first peak represents L-ß1-LAP fractions which eluted with sample 8-16 and the second peak represents the mature TGF-ß fraction.

The fractions containing rL-ß1-LAP were collected and then dialyzed against phosphate-buffered saline and finally filtered with a 0.22 µm membrane filter. The purified protein was then analyzed by SDS-PAGE. As shown in Figure 13, the purified rL-LAP migrated to a molecular weight of about 180 to about 200 kDa.

Antibodies specific to the LL-TGF-ß complex can be produced by introducing the complex to laboratory mice and screening the blood for response to the complex. Monoclonal antibodies may be produced by standard techniques.

The antibodies are useful for detection of the abnormal production of the LL-TGF-ß complex.

Mature TGF-ß is currently under study for use in the treatment of many diseases. Sporn et al., JAMA, 262: 938-941 (August 16, 1989); however mature TGF-ß1 has potent side effects such as body weight loss, anemia and thrombocytopenia. It is assumed that the use of LL-TGF-ß1 may be able to overcome some of these side effects.

TGF-ß1 is currently under study for use in the treatment of certain bone diseases, such as osteoporosis and bone fracture healing because TGF-ß is known to affect bone formation. TGF-ß is a potent inducer of type II collagen and proteoglycans, which form the extracellular matrix of cartilage. Sporn et al., supra. It has been

found that TGF-ß is one of the critical peptide growth factors that acts on chondrocytes or osteocytes and related cell types. Pfeilschifter et al., PNAS 84:2024-2028 (1987). It has been found to be involved in the embryonic formation of cartilage and bone and is present in the growth plates of long bones. Large amounts are also found in adult bone. In fact, TGF-ß2 was isolated from adult bovine bone.

LL-TGF-ß can also be useful as an immunosuppressant, in cases of autoimmune diseases or organ transplantation. TGF-ß is the most potent known endogenous suppressant of lymphocyte proliferation and function, and serves as an autocrine "stop" signal inhibiting the action of the interleukins and other cytokines, such as tumor necrosis factor, which stimulate lymphocyte function. Therefore, TGF-ß inhibits proliferation of T cells stimulated by interleukin 1 or 2, inhibits proliferation and antibody production in B cells stimulated by any of several activating factors, depresses cytolytic activity of natural killer cells, and inhibits the generation of cytotoxic T cells and lymphokine-activated killer cells. Sporn et al., supra. These effects have been confirmed in in vitro studies and in vivo studies are now in their early stages. These early tests show the suppression of cardiac allograft rejection across major histocompatibility barriers in mice. There are currently studies progressing in the use of TGF-ß in the areas of liver, pancreatic islet and kidney transplantation, and suppression of inflammatory processes driven by activated T cells in cases of experimental arthritis and clinical rheumatoid disease.

TGF-ß may also provide hematoprotection from cytostatic drugs by stem cell inhibition. Taking advantage of the immunosuppressive properties of LL-TGF-ß, patients, suffering from conditions for which chemotherapy is the treatment of choice, may be administered LL-TGF-ß prior to chemotherapy in order to protect cells of the bone marrow.

TGF-ß also has important actions on fibroblasts involved in tissue repair. TGF-ß stimulates the production of critical components of extracellular matrix, such as collagen, fibronectin and proteoglycans, and inhibits the action of proteolytic enzymes that destroy newly formed connective tissue. Sporn et al., J.Cell Biol. 105:1039-1045 (1987); Keski-Oja et al., J.Cell Biol. 106:451-459 (1987). Thus these actions result in the formation of new granulation tissue at the site of TGF-ß. Furthermore, TGF-ß has been shown to enhance gene transcription of collagen, Heine et al., J.Cell Biol. 105:2861-2876 (1987), and to play a critical role in providing the structural strength of healing wounds as well as serving as an essential part of the matrix of bone and cartilage. TGF-ß has been shown to enhance wound healing in animals. Roberts et al., Recent Prog. Horm. Res. 44:157-197 (1988). One such example of this is the stimulation of retinal reattachment in rabbits. Additionally, LL-TGF-ß1 may also be used to stimulate soft tissue healing, i.e. skin ulcers or peptic ulcers.

TGF-ß has been shown to be a potent antiproliferative agent for most epithelial cells and this may be of use as an antiproliferative agent in neoplastic diseases.

The large latency associated peptide (L-LAP) can be utilized as an antagonist for mature TGF-ß molecules. To ameliorate the possible toxic effects of TGF-ß treatment, L-LAP can be employed i.e., in therapeutic or diagnostic modalities, to control, modulate or regulate the harmful or undesired effects of TGF-ß treatment or presence. The use of L-LAP can overcome the drawbacks of TGF-ß therapy and can decrease or diminish the potent side effects of TGF-ß. The administration of L-LAP can also provide for a protective effect during treatment of other TGF-ß related diseases, i.e., fibrotic disorders, glomerulonephritis, liver fibrosis, keloid formation; and carcinoma metastasis.

Antibodies specific to the L-LAP can also be produced by introducing the peptide to laboratory animals and screening the blood for response to the complex. Monoclonal antibodies may be produced by standard techniques. These antibodies can be used as diagnostic aids.

The foregoing experiments show that it is possible to produce a recombinant LL-TGF-ß1 by introducing a DNA sequence for LTBP to a T23-7-11 CHO cell which expresses pro-TGF-ß1. By constructing a plasmid containing LTBP, and transfecting CHO cells with the plasmid, it is possible to express recombinant LL-TGF-ß1. The experiments also show that it is possible to produce a recombinant LL-TGF-ß2 by introducing a plasmid containing a cDNA sequence for TGF-ß2 into a BP-1-1 cell containing an amplified LTBP cDNA sequence.

The foregoing experiments also show that LTBP and LL-TGF-ß can bind Ca^{2+} and that by adding Ca^{2+} ions to the recombinant LL-TGF-ß complex produced results in increased protein stability and resistance against protease activity.

The preceding experiments also show that L-ß-LAP can be prepared from the rLL-TGF-ß complex by treating the complex with a denaturing agent to dissociate L-ß-LAP therefrom.

It is to be expected that the above-described methods of producing the recombinant LL-TGF-ß1 and LL-TGF-ß2 complexes can be applied generally to producing recombinant LL-TGF-ß complexes, including but not limited to recombinant TGF-ß3, because it is well documented that the individual members of the family of TGF-ßs are similar in peptide sequence and in biological activity. The TGF-ß isoforms exhibit comparable properties and thus are expected to behave similarly.

The experiments described herein suggest that the method for producing LL-TGF-ß complexes and related constructs may also be expressed in other eukaryotic cells,

using different plasmids and other vectors, the choice of which may be made by the skilled artisan.

It is believed that other embodiments may be incorporated into the present invention without departing from the spirit and scope of the invention. It is not intended that the present invention be limited to only the described embodiments. Modification of these embodiments will be recognized by those skilled in the art. Rather, the invention should be circumscribed by the scope of the appended claims.

CLAIMS

1. Method for producing large latent TGF-ß (LL-TGF-ß) comprising co-expressing in a transformed eukaryotic cell 1) a nucleic acid molecule which codes for LTBP and 2) a nucleic acid molecule which codes for pro-TGF-ß and culturing said cell under conditions favoring the production of large, latent TGF-ß.

2. Method of claim 1, wherein the TGF-ß is TGF-ß1.

3. Method of claim 1, wherein the TGF-ß is TGF-ß2.

4. Method of claim 1, wherein the TGF-ß is TGF-ß3.

5. Method for stabilizing LL-TGF-ß, comprising adding an amount of Ca^{2+} to an LL-TGF-ß containing sample in an amount sufficient to stabilize said LL-TGF-ß.

6. Method of claim 1, wherein said eukaryotic cell is a CHO cell.

7. Method of claim 2, wherein said eukaryotic cell is LT3-1.

8. Method of claim 3, wherein said eukaryotic cell is LT2-14.

9. Method of claim 1, comprising transforming said eukaryotic cell with a eukaryotic plasmid which contains at least one of said nucleic acid molecule which codes for LTBP and said nucleic acid molecule which codes for pro-TGF-ß.

10. Method of claim 2, comprising transforming said eukaryotic cell with a eukaryotic plasmid which contains at least one of said nucleic acid molecule

which codes for LTBP and said nucleic acid molecule which codes for pro-TGF-ß.

11. Method of claim 3, comprising transforming said eukaryotic cell with a eukaryotic plasmid which contains at least one of said nucleic acid molecule which codes for LTBP and said nucleic acid molecule which codes for pro-TGF-ß.

12. Method of claim 4, comprising transforming said eukaryotic cell with a eukaryotic plasmid which contains at least one of said nucleic acid molecule which codes for LTBP and said nucleic acid molecule which codes for pro-TGF-ß.

13. Method of claim 10, wherein said eukaryotic plasmid is pDSVE2-BP.

14. Method of claim 11, wherein said eukaryotic plasmid is pME-TGF-ß2.

15. Eukaryotic plasmid pME-TGF-ß2.

16. Method of treatment of a condition selected from the group consisting of osteoporosis, inflammation, and an immunological disorder comprising administering to a patient in need of said treatment a pharmaceutically effective amount of large latent TGF-ß1.

17. Method for treating a wound comprising administrating to a patient in need thereof a pharmaceutically effective amount of large latent TGF-ß1.

18. Method for inducing hematoprotection from cytostatic drugs by stem cell inhibition comprising treating a patient in need thereof a pharmaceutically effective amount of large latent TGF-ß1.

19. Method for treating neoplastic disease comprising treating a patient in need thereof with an amount of large latent TGF-ß1 sufficient to treat said neoplastic disease.

20. A cell line which produces LL-TGF-ß, having a heterologous DNA molecule which codes for LTBP and a heterologous DNA molecule which codes for pro-TGF-ß chromosomally incorporated therein.

21. The cell line of claim 20, wherein the TGF-ß is TGF-ß1.

22. The cell line of claim 21, wherein the cell line is LT3-1.

23. The cell line of claim 20, wherein the TGF-ß is TGF-ß2.

24. The cell line of claim 23, wherein the cell line is LT2-14.

25. Method for producing LTBP comprising expressing in a eukaryotic cell a heterologous DNA molecule which codes for LTBP, wherein said heterologous DNA molecule is chromosomally incorporated therein, and culturing said cell under conditions favoring the production of LTBP.

26. Method of claim 25, wherein said cell is a CHO cell.

27. Method of claim 25, wherein said eukaryotic cell is BP-1-1.

28. Cell line which produces LTBP, having a heterologous DNA molecule which codes for LTBP chromosomally incorporated therein.

29. Cell line of claim 28, wherein the cell line is BP-1-1.

30. Method for isolating L-LAP comprising,

(a) producing LL-TGF-ß by the method of claim 1,

(b) treating said LL-TGF-ß with a denaturing agent under conditions favoring dissociation of L-LAP therefrom; and

(c) separating L-LAP therefrom.

31. Isolated L-LAP having a molecular weight of about 180 to about 200 kDa as measured by SDS-PAGE.

32. Isolated L-LAP having a molecular weight of about 180 to about 200 kDa as measured by SDS-PAGE, which is obtained by

(1) producing LL-TGF-ß by the method of claim 1,

(2) treating said LL-TGF-ß with a denaturing agent under conditions favoring dissociation of L-LAP therefrom and

(3) separating L-LAP therefrom.

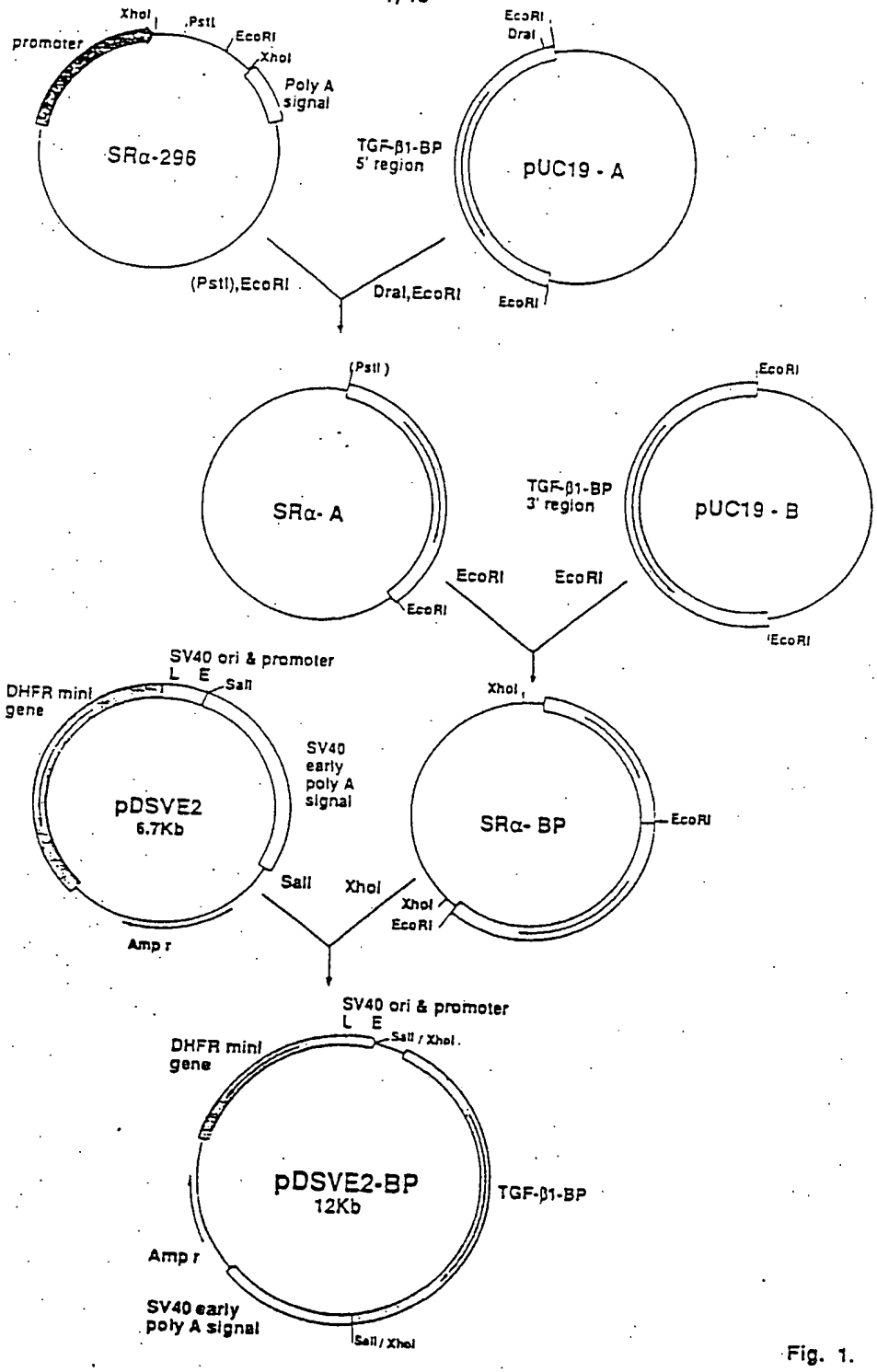

XhoI
PstI
promoter
EcoRI
XhoI
Poly A signal
SRα-296
TGF-β1-BP 5' region
EcoRI
DraI
pUC19 - A
EcoRI
(PstI),EcoRI
DraI,EcoRI
(PstI)
SRα- A
EcoRI
TGF-β1-BP 3' region
pUC19 - B
EcoRI
EcoRI
EcoRI
SV40 ori & promoter
L E
SalI
DHFR mini gene
pDSVE2 6.7Kb
SV40 early poly A signal
Amp r
SalI
XhoI
XhoI
EcoRI
SRα- BP
EcoRI
SV40 ori & promoter
L E
SalI / XhoI
DHFR mini gene
pDSVE2-BP 12Kb
TGF-β1-BP
Amp r
SV40 early poly A signal
SalI / XhoI

Fig. 1.

FIG. 2A

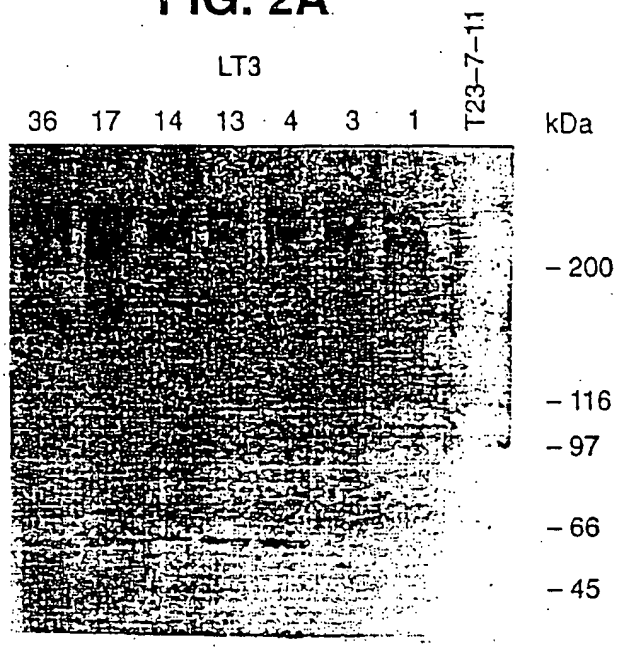

LT3
36 17 14 13 4 3 1
T23-7-11
kDa
200
116
97
66
45

FIG. 2B

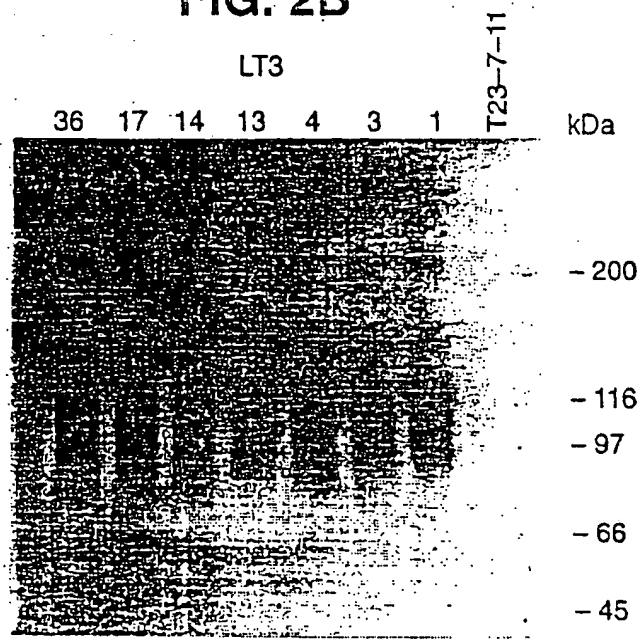

LT3
36 17 14 13 4 3 1
T23-7-11
kDa
200
116
97
66
45

FIG. 3

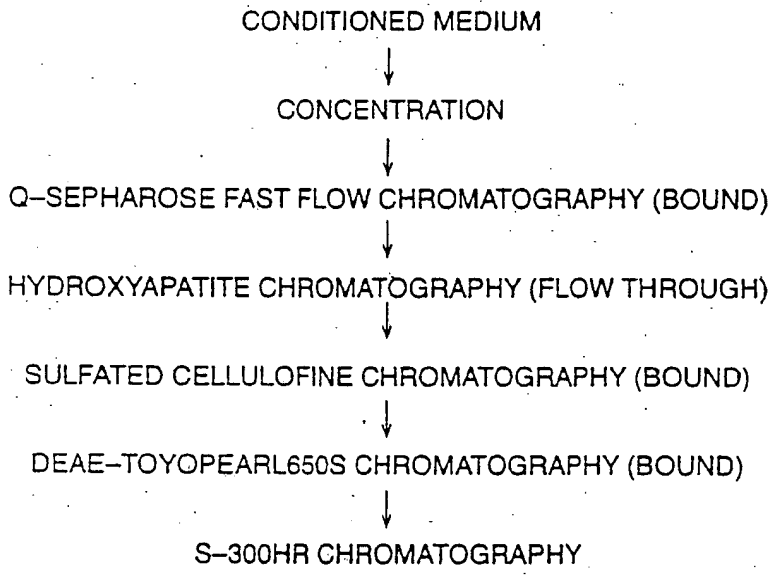

CONDITIONED MEDIUM
CONCENTRATION
Q–SEPHAROSE FAST FLOW CHROMATOGRAPHY (BOUND)
HYDROXYAPATITE CHROMATOGRAPHY (FLOW THROUGH)
SULFATED CELLULOFINE CHROMATOGRAPHY (BOUND)
DEAE–TOYOPEARL650S CHROMATOGRAPHY (BOUND)
S–300HR CHROMATOGRAPHY

FIG. 4C

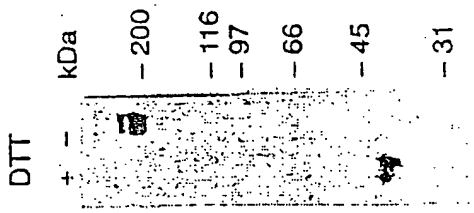


FIG. 4B

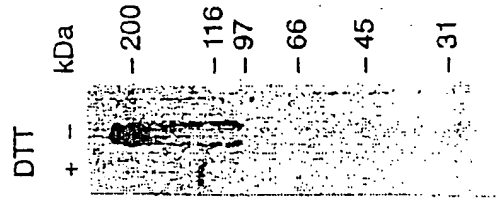


FIG. 4A

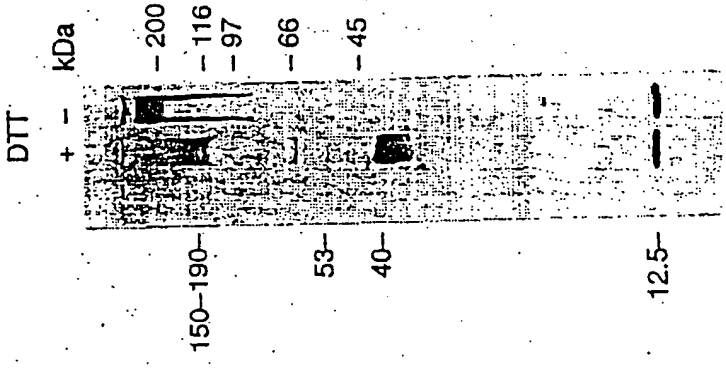


FIG. 5

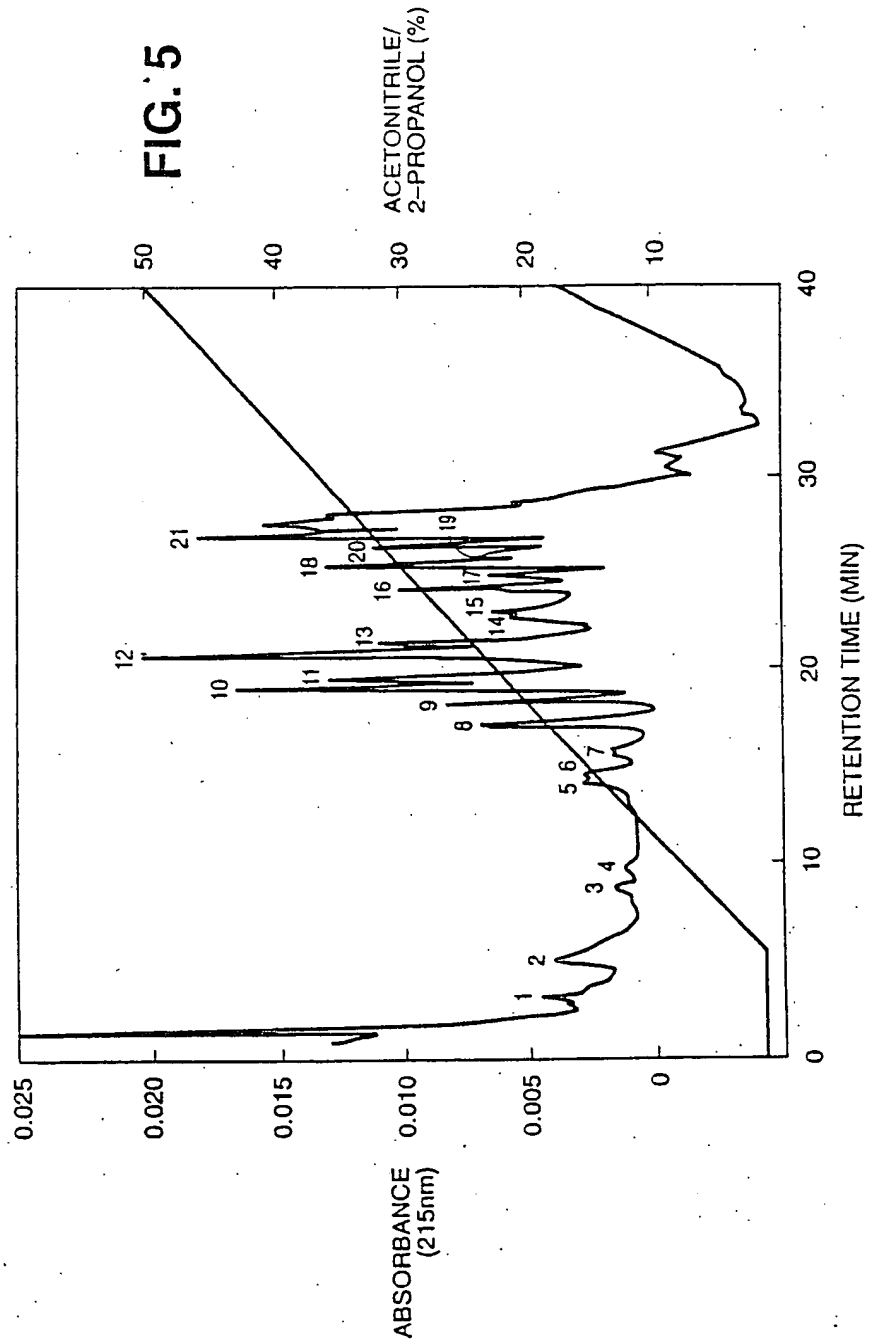

ACETONITRILE/
2-PROPANOL (%)
50
40
30
20
10
0
10
20
30
40
RETENTION TIME (MIN)
ABSORBANCE
(215nm)
0.025
0.020
0.015
0.010
0.005
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21

FIG. 6

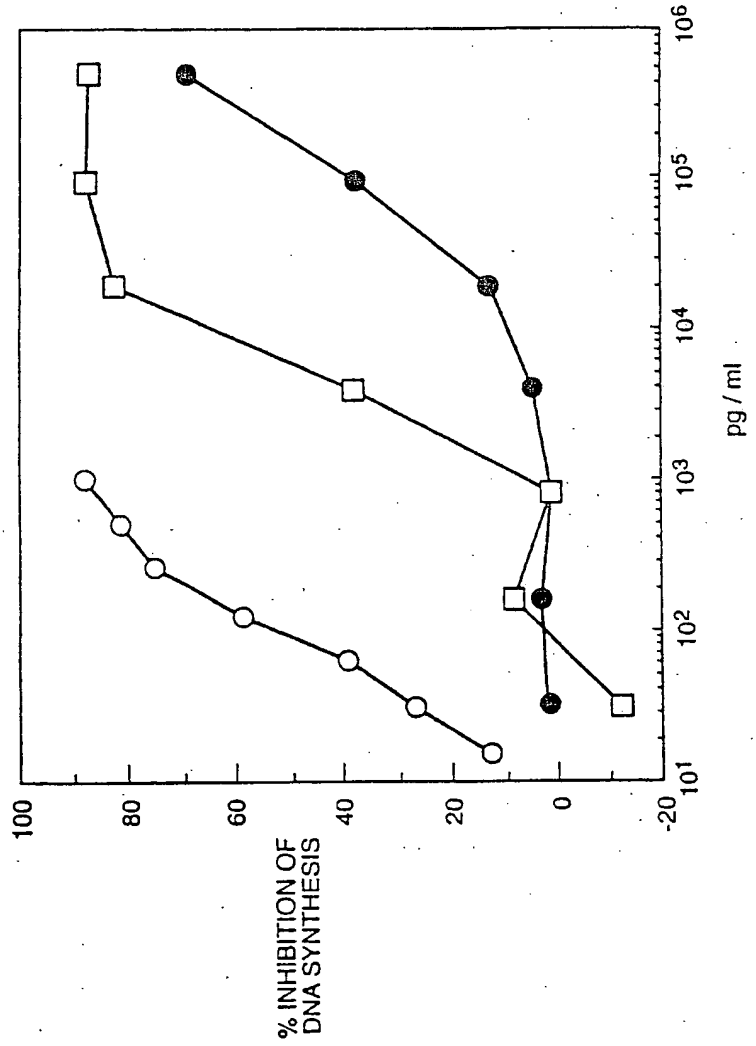

% INHIBITION OF DNA SYNTHESIS
100
80
60
40
20
0
-20
10^1
10^2
10^3
10^4
10^5
10^6
pg / ml

FIG. 7

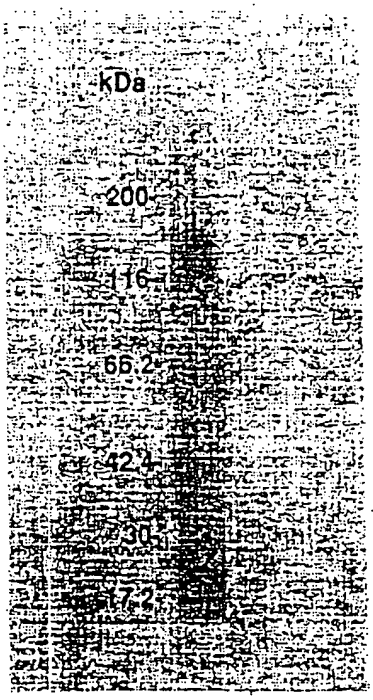


FIG. 8

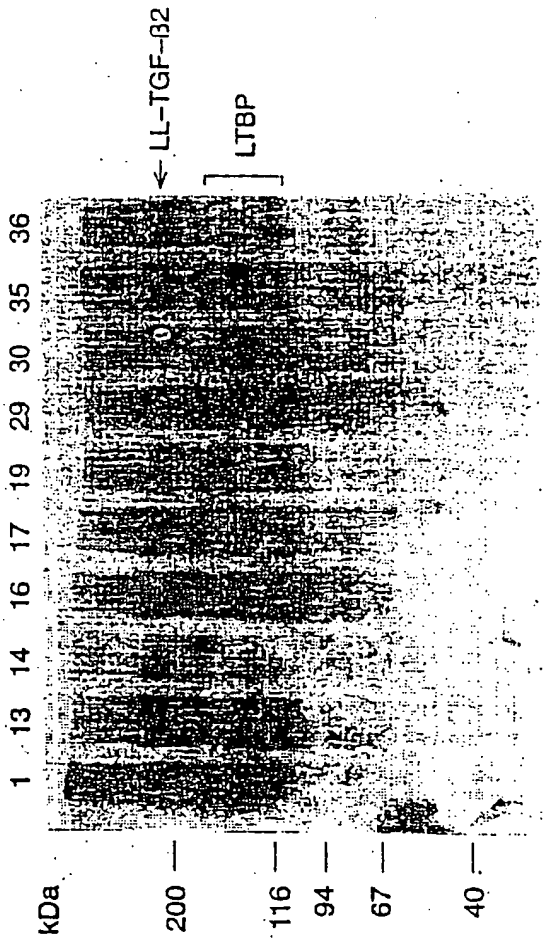

← LL-TGF-β2
LTBP
CELL CLONE
1
13
14
16
17
19
29
30
35
36
kDa
200
116
94
67
40

FIG. 9

	LL-TGF-β1					LTBP	SLC	B
EDTA (mM)	5	0	0	0	0	0	0	0
$CaCl_2$ (mM)	0	0	5	10	20	0	0	0

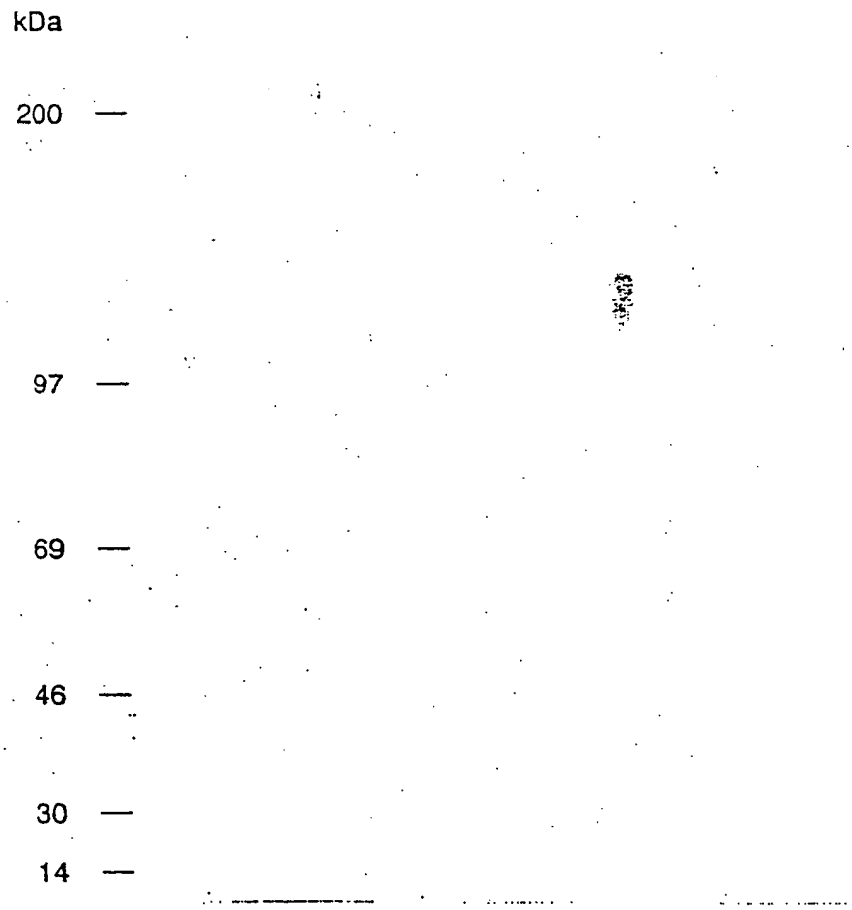


FIG. 10A

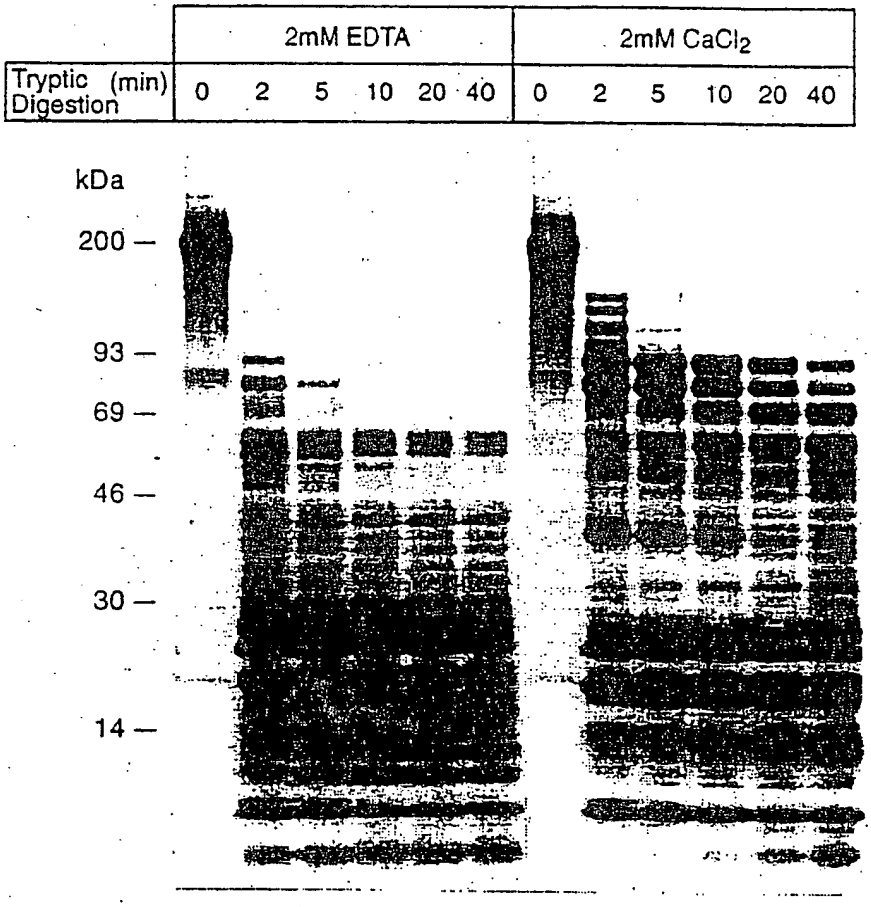
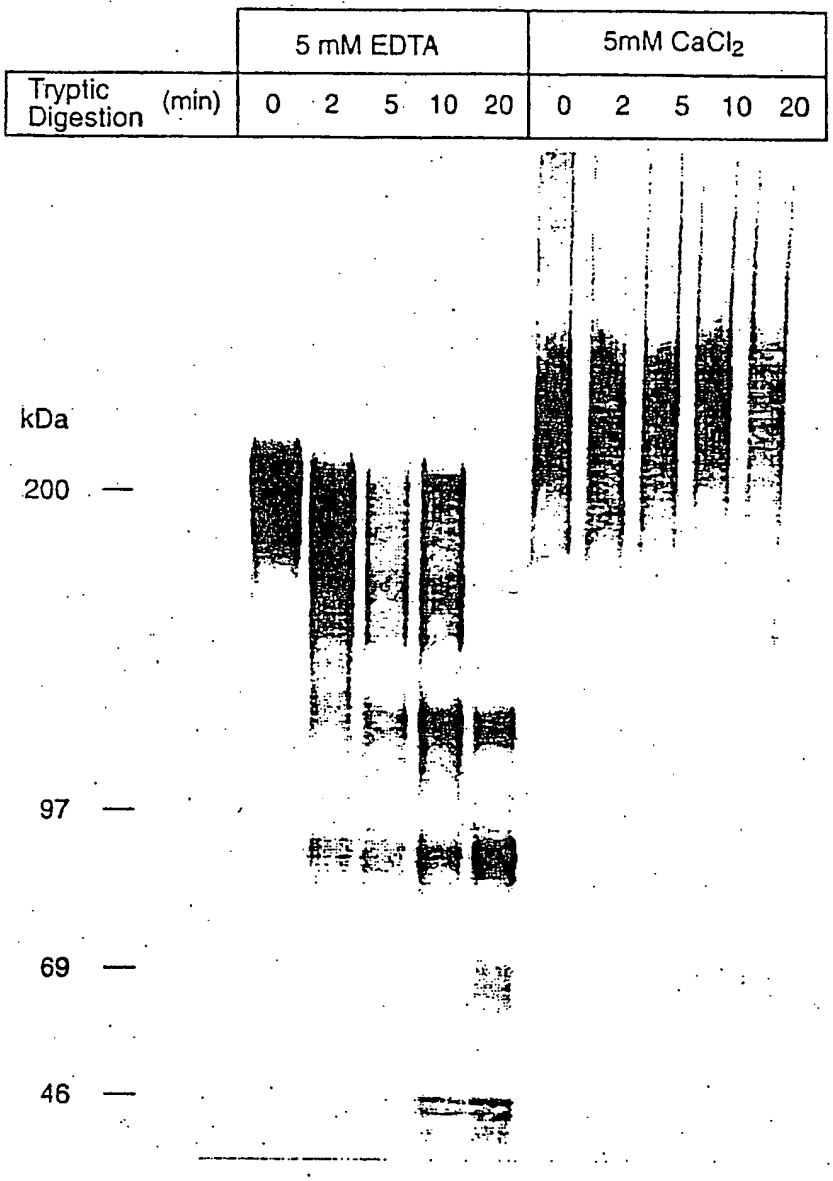

2mM EDTA
2mM CaCl2
Tryptic Digestion (min)
0 2 5 10 20 40
0 2 5 10 20 40
kDa
200
93
69
46
30
14

FIG. 10B


5 mM EDTA
5mM $CaCl_2$
Tryptic Digestion (min)
0 2 5 10 20
0 2 5 10 20
kDa
200
97
69
46

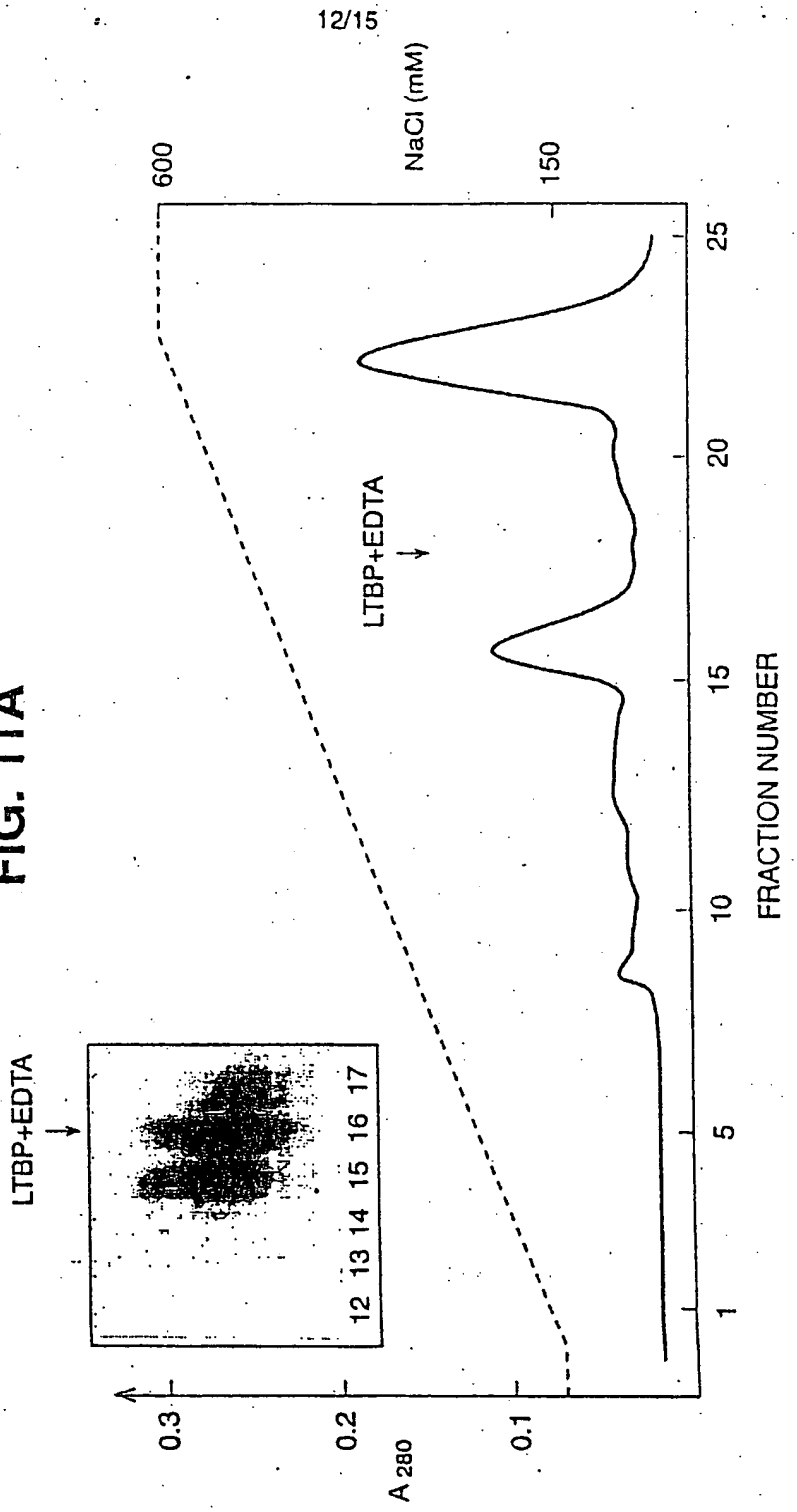

NaCl (mM)
600
150
LTBP+EDTA
25
20
15
10
5
1
FRACTION NUMBER
LTBP+EDTA
12 13 14 15 16 17
0.3
0.2
0.1
A 280

FIG. 11A

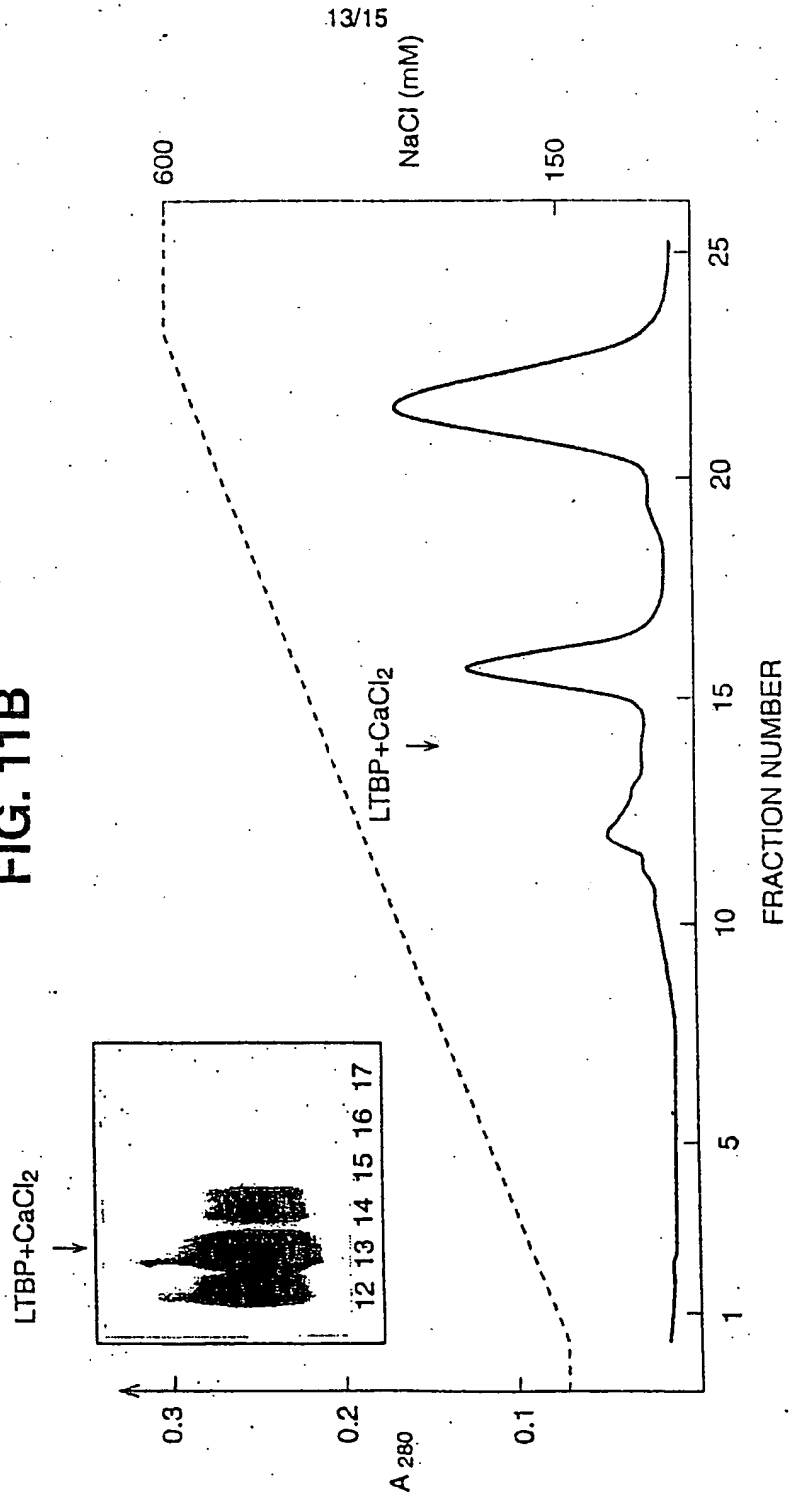

NaCl (mM)
600
150
LTBP+CaCl2
LTBP+CaCl2
12 13 14 15 16 17
0.3
0.2
0.1
A280
1
5
10
15
20
25
FRACTION NUMBER

FIG. 11B

FIG. 12

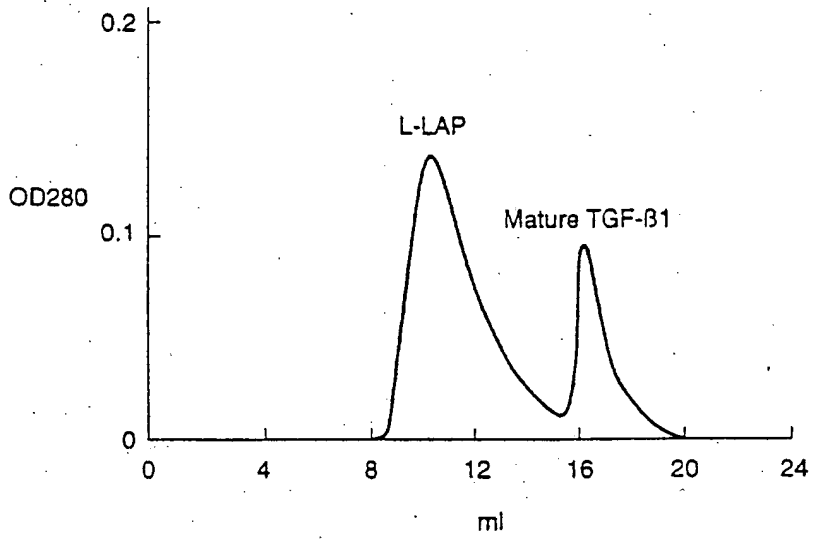


FIG. 13

INTERNATIONAL SEARCH REPORT

Inter_tional application No.
PCT/US93/10230

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/36; C07K 13/00
US CL :530/399; 435/69.1, 69.4, 68.8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/399; 435/69.1, 69.4, 68.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Vol. 61, issued 15 June 1990, T. Kanzaki et al., "TGF-beta 1 binding protein: A component of the large latent complex of TGF-beta 1 with multiple repeat sequences", pages 1051-1061.	1-32
Y	EMBO J, Vol. 10, No. 5, issued May 1991, K. Miyazono et al., "A role of the latent TGF-beta 1 binding protein in the assembly and secretion of TGF-beta 1", pages 1091-1101, see especially page 1100, paragraph 1.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 December 1993	Date of mailing of the international search report 04 FEB 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer KAREN COCHRANE CARLSON, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10230

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Growth Factors, Vol. 1, issue 1989, L.M. Wakefield et al., "Recombinant TGF-beta 1 is synthesized as a two-component latent complex that shares some structural features with the native platelet latent TGF-beta 1 complex", pages 203-218.	1-25
Y	Growth Factors, Vol. 3, issued 1990, P.D. Brown et al., "Physiochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3", pages 35-43.	3, 4, 6, 8, 11, 13, 14, 22, 23
Y	J. Sambrook et al., Molecular Cloning, A Laboratory Manual, published 1989 by Cold Spring Harbor Laboratory Press (NY), see pages 16.30-16.36.	26-32